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Distinct roles of HF-1b/Sp4 in ventricular and neural crest cells lineages affect cardiac conduction system development

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Abstract

The heterogeneous cell types of the cardiac conduction system are responsible for coordinating and maintaining rhythmic contractions of the heart. While it has been shown that the cells of the conduction system are derived from myocytes, additional cell types, including neural crest cells, may play a role in the development and maturation of these specialized cell lineages. Previous work has shown that the expression of the *hf-1b* gene is required for specification of the cardiac conduction system. Using Cre-Lox technology, we conditionally mutated the *hf-1b* gene in the ventricular and the neural crest cell lineages. Cx40 immunohistochemistry on HF-1b tissue-restricted knockouts revealed a requirement for HF-1b in the cardiomyogenic lineage. Electrophysiological studies identified a second requirement for HF-1b in the neural crest-derived cells. Absence of HF-1b in the neural crest led to atrial and atrioventricular dysfunction resulting from deficiencies in the neurotrophin receptor trkC. Therefore, in this study, we document that a single transcription factor, HF-1b, acts through two separate cell types to direct distinct functions of the cardiac conduction system.

Keywords: Heart failure; Conduction system; Cardiac development; Neural crest

Introduction

Contractile rhythm of the heart depends on the coordinated function of the heterogeneous cell lineages comprising the cardiac conduction system. These cell lineages are organized into a network of specialized pacemaking components, including the sinoatrial (SA) node, the atrioventricular (AV) node, His-bundle, and Purkinje fiber complex, which are necessary for propagation of electrical impulses through the heart. Each component of the cardiac conduction system, as well as the myogenic cells themselves, is unique and distinct in regard to their cellular structure (Viragh and Challice, 1981) and electrophysiological properties (Munk et al., 1996). Disruption of the development, differentiation, or maturation of any of these components can lead to arrhythmic anomalies such as sinus arrest, AV block, ventricular tachycardia, and sudden death (Bruneau et al., 2001; Nguyen-Tran et al., 2000).

Until recently, the precise identification of these specialized cells of the cardiac conduction system as well as an understanding of the molecular mechanisms behind the development and differentiation of these cells has remained unclear. Of particular interest is the origin of the conduction system lineages. The first major step towards elucidating the source of the conduction cells came from retroviral lineage tracing studies in the chick (Cheng et al., 1999; Gourdie et al., 1995). These studies provided compelling evidence that conduction cells are derived from cardiomyogenic precursors of the linear heart tube. In addition, evidence points to a potential role for paracrine signals derived from non-muscle

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cell types in the development and differentiation of these specialized lineages. For example, studies in the avian system have implicated signals originating from the coronary arteries as necessary for ventricular conduction cell differentiation (Hyer et al., 1999; Poelmann and Gittenberger-de Groot, 1999), and endothelin treatment can induce the conversion of embryonic myocytes into impulse-conducting Purkinje fibers (Gourdie et al., 1998).

Although our understanding of the precise molecular mechanisms that trigger arrhythmogenesis and sudden death remain unclear, recent studies have begun to identify key components in the sudden death pathway (Bruneau et al., 1999; Kuo et al., 2001; Nguyen-Tran et al., 2000; Schott et al., 1998), these include transcription factors, signaling molecules, channels, and channel regulators (Chien and Olson, 2002; Pashmforoush et al., 2001). In this regard, we previously reported that mice mutant for the *hf-1b* gene exhibit a high incidence of postnatal mortality and are susceptible to rhythm disturbances. These disturbances include aberrant ventricular myocyte excitability as well as sinus bradycardia and AV block, although these mice display normal cardiac morphology and contractile function (Nguyen-Tran et al., 2000).

The *hf-1b* gene encodes an SP1-related transcription factor and is preferentially expressed in the conduction system and ventricular myocytes of the mouse heart, as well as in the brain and neural tube (Nguyen-Tran et al., 2000; Zhu et al., 1993). The neural tube gives rise to the neural crest, a transient cell lineage that migrates into a number of regions in the embryo including the nervous system and the heart. In the heart, the cardiac neural crest cells migrate to the endocardial cushion and outflow tract (Jiang et al., 2000). The neural crest is also important for the formation of the parasympathetic postganglionic innervation of the heart that preferentially innervates the atrioventricular node (AVN) and, to a lesser extent, the sinus atrial node (SAN) and the His-bundle (Nakagawa et al., 1993; Verberne et al., 1998). Survival of these innervating neurons is partially dependent on the neurotrophin NT-3 and its interaction with the Trk receptor tyrosine kinases (Story et al., 2000; Tessarollo et al., 1997).

Though the neural crest is not directly involved in the development of the conduction system, studies have shown that cardiac neural crest may play a role in the late phase of cardiac conduction system cell maturation (Poelmann and Gittenberger-de Groot, 1999) and thus may be necessary for its development. In this paper, we identify key developmental components necessary for the specification and function of cardiac conduction, in particular with dissect the specific roles of HF-1B in the ventricular myocardium and in neural crest cells upon conditional mutagenesis of HF-1b in these compartments.

Material and methods

Generation of the HF-1b floxed allele

The organization of the HF-1b genomic locus has been previously described (Nguyen-Tran et al., 2000). The HF-1b floxed-targeting vector was generated by PCR amplification. 2.1 kb of the genomic sequence, including exon 3, was amplified using primers containing BamH1 restriction sites. The fragment was then cloned to the BamH1 site in the pFlox vector flanked by two loxP sites. 3.8 kb of upstream sequence was cloned into a unique Xho1 site, while 6 kb of downstream sequence was cloned into the unique Xba1 site 3′ to the TK/neo cassette. The targeting construct was linearized using Not1. Two G418 resistant ES clones were identified as homologous recombinants based on Southern blot hybridization analysis, and one clone was subsequently transfected with cre-recombinase. Type I and Type II recombinants were screened for by Southern blot analysis, and two Type II clones were selected for injection to pseudo-pregnant mice. Chimeric male mice were crossed with Black Swiss breeders.

Generation of HF-1b conditional knockouts

HF-1b floxed mice were crossed with mice transgenic for cre-recombinase. Mice harboring the protamine-cre transgene were used to generate the global knockout. Male mice positive for protamine-cre and HF-1b floxed allele were crossed with Black Swiss breeders, and heterozygous offspring were used for further breeding to generate mice with the HF-1b mutation in the germ line. Tissue restricted knockout mice were generated using a variety of transgenic lines. MLC2v Cre mice were used to generate the ventricular restricted knockout. Pax3 Cre mice were used to generate the neural crest restricted lines. Male mice from these crosses were crossed with homozygous HF-1b floxed animals to generate animals homozygous for the tissue-restricted mutation of HF-1b.

Histochemical analysis and imaging

Enzymatic histochemical detection of the β-galactosidase activity on whole embryos was performed using previously described protocols (Nguyen-Tran et al., 2000; Ross et al., 1996). Neuronal Class III β-tubulin immunohistochemistry was performed using a diluted 1:50 monoclonal antibody (MMS-435P, Babco) followed 1:200 biotinylated anti-mouse (Vector), 1:1000 streptavidin-β-galactosidase (Sigma) and developed by β-galactosidase detection. Adult hearts from mice in which nuclear β-galactosidase was knocked into the HF-1b locus were provided by Dr. Xianjin Zhou (K. R. Chien unpublished). Sections from Pax3-cre mice crossed with Rosa26R were stained as described previously (Li et al., 2000). Acetylcholinesterase (AChE) staining was performed according to (El-Badawi and Shenk, 1967).

Immunohistochemical analysis of Cx40 was performed as follows: frozen sections were acetone fixed for 10 min, incubated with a 1:50 dilution of a rabbit anti-mouse Cx40 antibody (Chemicon) and visualized with anti-rabbit-Alexa Fluor 488 (Molecular Probes). After 30 min of postfixation in 4% paraformaldehyde, cell outlines were labeled with wheat germ agglutinin 1:50 dilution (Molecular Probes). All fluorescent immunohistochemical experiments were captured with a DeltaVision deconvolution microscope system (Applied Precision, Inc., Issaquah, WA). Optical sections were taken in increments of 0.2–0.5 um depending on the magnification. Exposure times were set such that the camera response was in the linear range for each fluorophore. The data sets were deconvoluted and analyzed using SoftWorx software (Applied Precision, Inc) on a Silicon Graphics Octane workstation. Cx40 distribution was scored upon delineation of the cell margins, using in a rhodamine-conjugated wheat germ agglutinin. We scored Cx40 distribution in distal Purkinje fibers as follows: number of positive immunofluorescence signal not co-localizing with cell membrane/total positive immunofluorescence signal, where 'n' is the number of different experiments ($n = 6$) with a count of 250–350 immunofluorescence signals per experiment. The measurements are statistically significant as shown by t test. Data are shown as mean ± SEM $*P \leq 0.05$.

Intracardiac electrophysiology by programmed stimulation

Standard protocols for programmed stimulation and electrophysiological studies in mice were completed as described previously (Kuo et al., 2001). Adult mice ranging in age from 6 weeks to 15 months were anesthetized intraperitoneally with pentobarbital (initial dose of 0.033 mg/g with supplemental doses of 0.016 mg/g as necessary) and ketamine, and a double lead surface ECG was obtained. Rapid atrial pacing techniques were performed to determine sinus node recovery time and antegrade AV node conductivity.

Corrected sinus node recovery time (cSNRT) intervals were determined from measurements of the sinus node recovery time (SNRT) minus the normal sinus cycle length (SCL). This was followed by an extra stimulation (S1S2) pacing technique to determine atrial effective refractory period (AERP). Ventricular pacing using the same protocol followed immediately. Measurements were made at 200, 150, 120 and 100 ms. Electrophysiological variables were subjected to one-way ANOVA analysis (Prism ver4.0, GraphRad). Variables with overall P value under 0.05 were subjected to a post hoc test (Student–Neuman–Keuls) to evaluate differences between all paired groups. Although changes are small, statistic analysis indicates null hypothesis is wrong between measured groups with chances of $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

RT-PCR

Total RNA was isolated from whole hearts of age-matched mice. Tissue was homogenized in Trizol reagent to isolate total RNA. Reverse transcription was performed followed by a 30-cycle PCR reaction using gene specific primers. Gel band quantification of HF-1b in wild type and knockout animals was performed by densitometry analysis using a gel imaging system.

Results

Generation of HF-1b floxed mice

A systemic knockout of HF-1b that displays sudden cardiac death and conduction system defects has been previously described (Nguyen-Tran et al., 2000). In order to evaluate the specific role of HF-1b in cardiac conduction system, we have generated an *hf-1b* floxed allele (Figs. 1A, B) that we have used for mutagenesis in the ventricular myocytes and in neural crest derivatives. Mice heterozygous for the HF-1b floxed allele (HF-1b$^{f/+}$) were crossed to generate homozygous HF-1b floxed (HF-1b$^{f/f}$) animals (Fig. 1C).

To establish whether the *hf-1b* floxed allele was capable of generating a phenotype similar to the conventional gene targeted HF-1b$^{-/-}$ mice, we crossed HF-1b$^{f/f}$ animals with mice expressing *cre*-recombinase in the male germline under the control of the *protamine* promoter (O'Gorman et al., 1997). Semi-quantitative RT-PCR performed on mutant adult whole hearts revealed complete efficiency of the protamine promoter and the complete deletion of HF-1b in the global knockout mice (Fig. 1D). Phenotypically, global HF-1b knockout mice survived to birth but were much smaller than their wild type and heterozygous littermates. Furthermore, these mice displayed an increased incidence of perinatal death (4/24, 16.7%), similar to the perinatal death observed in the global KO (Nguyen-Tran et al., 2000). Thus, the HF-1b floxed allele was capable of being conditionally mutated to a true null phenotype in vivo via Cre-lox strategies, supporting the utility of these mice for tissue restricted ablation studies (Fig. 1C).

Generation of HF-1b conditional KO lines

To dissect the role of myocyte anomalies versus direct neuronal influences on the developing conduction system and the potential for arrhythmogenesis in the HF-1B mouse, we generated conditional knockouts of HF-1b from crosses of HF-1b$^{f/f}$ mice with transgenic mice in which the Cre-recombinase gene was under the control of two different lineage specific

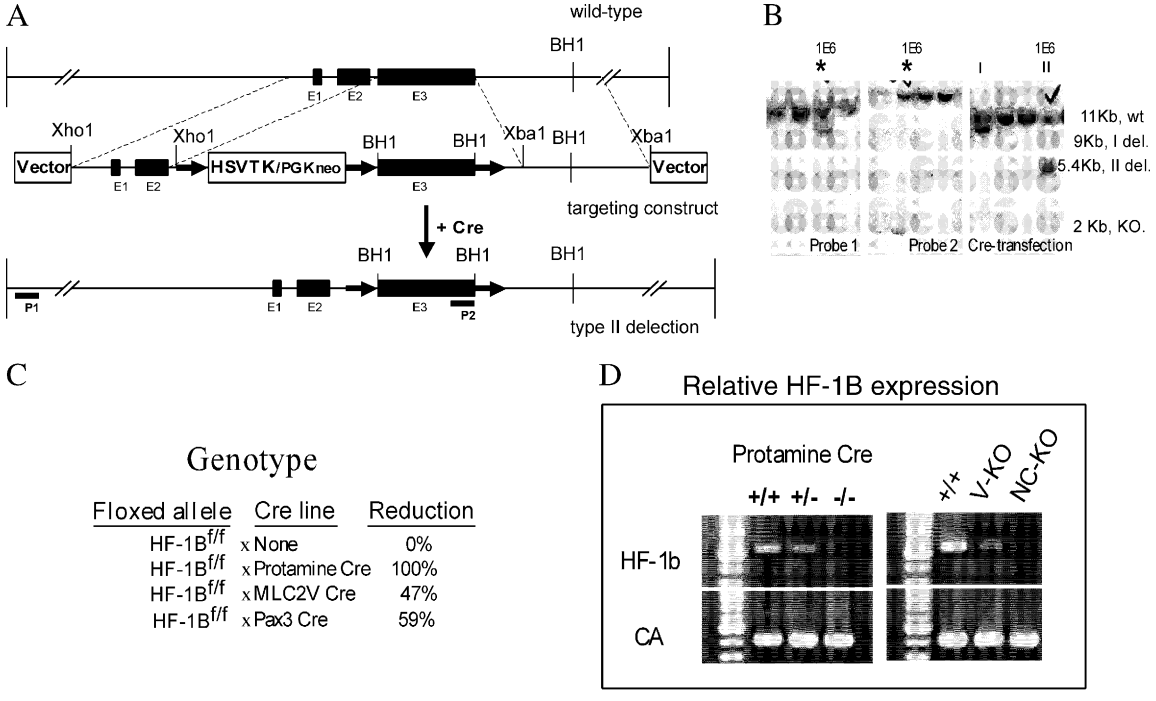


Fig. 1. Gene targeting strategy for HF-1b floxed allele. (A) Diagram of the *HF-1b* endogenous locus, the targeting construct and the resulting floxed HF-1b allele. Exons are denoted as solid black boxes (E1, E2 and E3). Two separate probes were used to screen for the targeted allele as denoted by the solid black lines (P1 and P2). (B) Representative Southern blots from the three ES cell screens using P1 and P2 probes and from the second ES cell screen after cre-recombinase transfection. Clones were screened for Type I and Type II deletions, and only Type II deletions were used for mouse injection. (C) Generation of HF-1b conditional knockouts from crosses with various transgenic cre mouse lines. (D) RT-PCR for *HF-1b* from adult whole hearts isolated from wild type, heterozygous and conditional mutant lines. Cardiac actin was used as an internal control (CA).

promoters specifying either the ventricular muscle or neural crest (Fig. 1C). To generate a ventricular restricted knockout of HF-1b (V-KO), we crossed HF-1b$^{f/f}$ female mice with mice carrying Cre-recombinase under the control of the myosin light chain 2v (*MLC2v*) promoter (Chen et al., 1998). *MLC2v* is the earliest ventricular-restricted marker during mammalian cardiogenesis and displays a high efficiency for ventricular restricted targeting (Chen et al., 1998). The efficiency of conditional gene ablation was measured by semi-quantitative RT-PCR, which revealed a 47% reduction in *hf-1b* expression in the whole adult heart (Fig. 1D).

MLC2v is expressed bilaterally in the ventricular segment of the myocardium and remains restricted to the ventricular myocytes. Recent studies have shown *MLC2v* expression in several components of the conduction system, but primarily in the AVN (Franco and Icardo, 2001). Using a nuclear MLC2v β-galactosidase indicator system, we have also observed expression of MLC2v that is localized to a region of the right atria (Fig. 2A). This region is ventro-medial to the right sinus horn and in the region of the future SA node (Heintzberger, 1974), a location that was subsequently confirmed by in situ hybridization (Figs. 2B and C). Therefore, the ventricular-restricted deletion of HF-1b reaches all components of the conduction system including the SA node.

The neural crest restricted knockout of HF-1b (NC-KO) was generated from crosses of HF-1b$^{f/f}$ female mice with mice carrying *cre*-recombinase under the control of the *Pax3* promoter (Li et al., 2000). *Pax3* is expressed early during development in the dorsal neural tube and somites and has been shown to be important for proper development of these tissues (Goulding et al., 1991). The efficiency for this promoter has been extensively described and restricts the cardiac expression of Cre-recombinase to the dorsal neural tube, the cells invading the outflow tract of the heart and the dorsal root and sympathetic ganglia (Epstein and Buck, 2000; Epstein et al., 2000; Li et al., 2000). Semi-quantitative RT-PCR analysis of whole adult hearts from NC-KO animals revealed a 59.22% reduction in the level of HF-1b expression (Fig. 1D).

To confirm the specific localization of Cre to the neural crest-derived cells in the heart and to exclude *cre* expression in the myocytes, the ROSA26R indicator mouse was crossed into the Pax3-cre line. Sections through embryonic day (E) 16.5 and adult hearts (Figs. 2D and E respectively) showed that β-galactosidase (blue) staining is substantially absent in the myocardium while significant β-galactosidase staining was detected in the smooth muscle cells of the ascending and descending aorta (Fig. 2F). We did not detect staining in components of the conduction system.

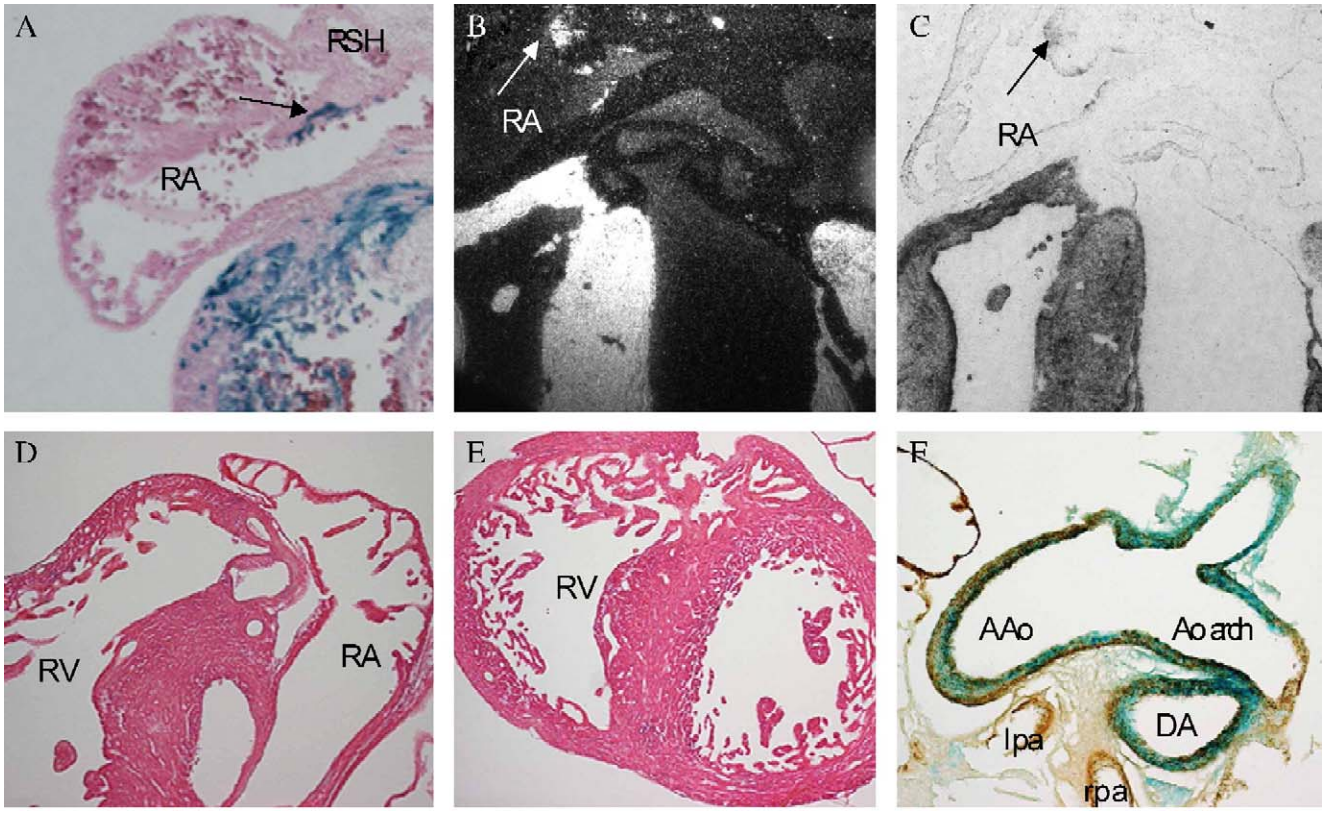
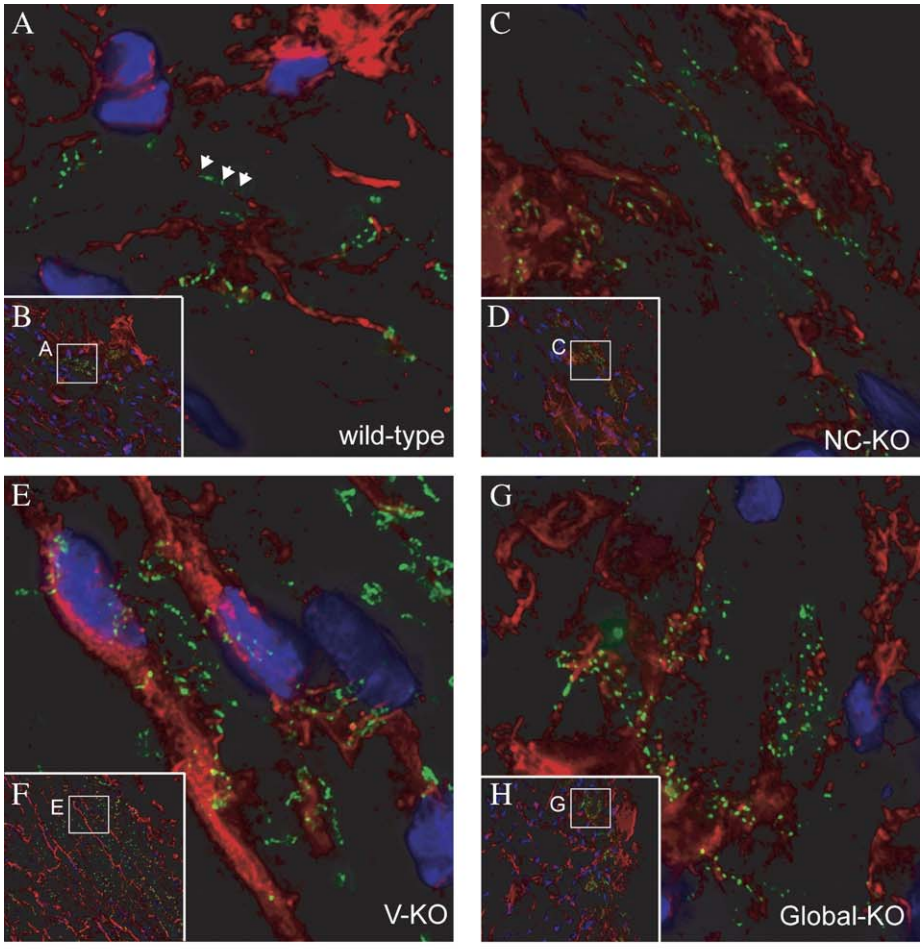


Fig. 2. β-Galactosidase staining reflects the localization of cre expression in the *MLC2*-cre and *Pax3*-cre mice. (A) β-Galactosidase staining from a cross-section of E15.5 mouse heart confirming the expression of cre-recombinase under the control of the *MLC2v* promoter in the SA node. (B and C) Section in situ from D4 mouse heart showing *MLC2v* expression in the presumptive SA node region of the mouse heart. Dark field (A) and light field (B). (D–F) β-Gal staining of sections from E16.5 (D) and adult (E and F) hearts confirm the absence of leaky cre expression in the myocardium. (F) Smooth muscle and β-galactosidase co-staining of the ascending aorta and aortic arch at the level of the ductus arteriosus. RA, right atria; RV, right ventricle; RSH, right sinus horn; Aao, ascending aorta; Ao arch, aortic arch; DA, ductus arteriosus; lpa, left pulmonary artery; rpa, right pulmonary artery.

Phenotypic analysis of HF-1b conditional knockout animals reveals a confused identity of the peripheral conduction system

The adult conduction system comprises the sinoatrial (SAN), and atrioventricular (AVN) nodes, the atrioventricular bundle (AVB), the bundle branches and the peripheral Purkinje fibers, each of which displays distinct functional properties and distinct profile of gene expression (Franco and Icardo, 2001). Previous studies (Nguyen-Tran et al., 2000) have shown that systemic HF-1b mutation leads to abnormal



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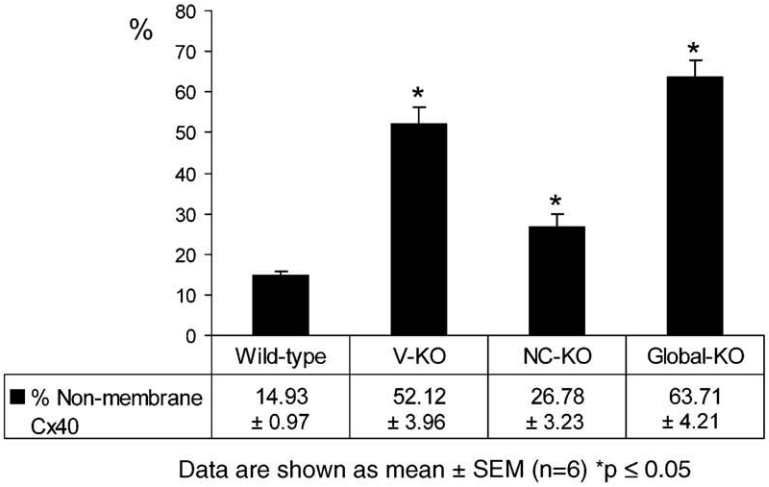

	Wild-type	V-KO	NC-KO	Global-KO
■ % Non-membrane Cx40	14.93 ± 0.97	52.12 ± 3.96	26.78 ± 3.23	63.71 ± 4.21

Data are shown as mean ± SEM (n=6) *p ≤ 0.05

Fig. 3. Expression of Connexin 40 (Cx40) in the distal Purkinje fibers of wild type (A, B), NC-KO (C, D), V-KO (E, F) and global HF-1b KO (G, H) adult mice. Membranes were stained with wheat germ agglutinin (in red). Images and measurements are taken from the subendocardial area of the ventricular wall in which Purkinje fibers run through the sub-endothelial connective tissue. Low magnifications (B, D, F, H) are shown to demonstrate the subendocardial location of the cells scored. Calculated disorganization of Cx40 expression at the cell borders as described in Materials and methods (I). Data is shown as mean ± SEM *$P \leq 0.05$.

localization of Connexin 40 (Cx40), which is a marker for the cardiac Purkinje cells (Delorme et al., 1995; Gourdie et al., 1993).

To analyze the phenotype of the HF-1b conditional knockout lines, we performed Cx40 immunohistochemistry (Fig. 3). Cx40 localized to the proximal region of the His bundle, bundle branches (data not shown) and to the peripheral Purkinje fibers in all conditional knockout lines (Figs. 3A–C). We noticed a marked disorganization of Cx40 intracellular distribution that was dependent upon the HF-1b genotype. In wild-type hearts, Cx40 staining appeared to be organized along the cell boundaries. NC-KO animals also displayed relative maintenance of organization among cells of the peripheral conduction system. However, the cellular distribution of Cx40 in the V-KO animals was diffuse (Fig. 3). To further quantify the organization of Cx40 in the different HF-1b mutants, we scored for Cx40 distribution in the Purkinje fibers by dividing the Cx40 signal localized along the cell membrane by the total Cx40 immunofluorescence signal (Fig. 3I). In the wild type, Cx40 staining appeared organized along the cell boundaries, as indicated by co-localization with wheat germ agglutinin (disarray:14.93 ± 0.97). NC-KO animals displayed significant disorganization among cells of the peripheral conduction system (disarray: 26.78 ± 3.23). Compared to wild type and NC-KO animals, the cellular distribution of Cx40 in the V-KO animals was dispersed throughout the cell. Only 47% of Cx40 staining was found along the cell border (disarray: 52.12 ± 3.96) compared to a disarray score of 63.71 ± 4.21 disarray found in the global KO mice (Fig. 3I). In other words, over half of the Cx40 positive signal was distributed throughout the cellular cytoplasm in the V-KO animals. These data point to an intrinsic requirement for HF-1b in the ventricular myocyte lineage, which is necessary for Cx40 distribution in the Purkinje fibers.

Electrophysiological analysis of HF-1b conditional knockouts reveals a requirement for HF-1b in the neural crest for proper functioning of the cardiac conduction system

To analyze the electrical phenotypes of the diverse *Hf-1b* mutants, we carried out a program stimulation protocol involving rapid atrial and ventricular pacing techniques (Berul et al., 1996; Kuo et al., 2001). Programmed stimulation measurements on global knockout mice compared to wild-type controls provided the baseline phenotype (Table 1). Atrial pacing of the HF-1b global knockout mice resulted in significant increases in both Wenckebach cycle length and 2:1 atrioventricular block cycle length. Atrial effective refractory period (AERP) was also significantly longer in the global knockout animals as compared to wild-type littermates. However, no significant susceptibility to induction of ventricular tachycardia was observed upon ventricular premature stimulation. Therefore, electrophysiological defects of HF-1b global knockout mice are localized to the atrial and atrioventricular components of the cardiac conduction system.

Program stimulation data from V-KO mice showed no significant electrophysiological defects among the parameters analyzed (Table 1). Interestingly, conduction defects were prominent in NC-KO animals. As shown in the global and V-KO mouse lines, no ventricular arrhythmias were induced in these animals by premature stimulation. However, atrial paced 2:1 AV block rates were significantly higher in NC-KO animals as compared to wild type (Table 1). In addition, AERP elongation found in the global knockout mice was recapitulated in NC-KO animals. Therefore, HF-1b expression in the neural crest plays a necessary role for atrial and atrioventricular conduction system function.

Table 1
Intracardiac electrophysiological analysis of cardiac conduction system function by program stimulation

Pacing parameters (ms)	SNRT	SCL	CSNRT	AP Weak	2:1 block	AERP	VERP
Wild type (n=10)							
Mean	156.50	136.36	52.80	79.00	60.00	53.33	44.00
SD	33.94	31.14	29.57	9.94	8.16	10.00	10.75
Global KQ (n=7)							
Mean	166.00	143.00	54.00	95.71**	74.29**	80.00***	51.43
SD	32.88	27.55	18.51	7.87	5.35	11.55	21.16
Ventricular KQ (n=11)							
Mean	165.30	141.17	50.4	80.00	62.73	61.82	54.00
SD	30.25	18.58	17.60	10.00	11.04	13.28	16.47
Neural Crest KQ (n=11)							
Mean	189.09	145.63	63.64	86.36	69.09*	70.00**	46.00
SD	50.33	30.43	24.03	6.74	7.01	15.49	14.30

Wild type, global, V-KO, and NC-KO mice were analyzed for electrophysiological defects using a standard program stimulation protocol. Rapid atrial pacing techniques were performed to determine sinus node recovery time, Weinkebach cycle length, atrial 2:1 conduction block, atrial effective refractory period (AERP), and susceptibility of induced atrial arrhythmias. Ventricular pacing followed using the same protocol. All interval values are in milliseconds (ms). SNRT, sinus node recovery time; SCL, sinus cycle length; CSNRT, corrected sinus node recovery time; APWenk, atrial pacing Wenckeback cycle length; 2:1 block, atrial pacing 2 to 1 conduction block; AERP, atrial effective refractory period; VERP, ventricular effective refractory period. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

HF-1b is a regulator for trkC and results in a decrease of innervation to the AV node

Neurotrophins play a crucial role in the development of the peripheral nervous system. In particular, NT-3 (neurotrophin-3) is expressed in part of the developing cardiac conduction system (Hiltunen et al., 1996) and is a downstream component of the HF-1b pathway in the brain (Zhou et al., 2005). To determine if HF-1b deficiencies in the heart lead to changes to *NT-3* expression, we performed RT-PCR experiments on adult hearts. Unlike the effect seen in the brain, NT-3 levels were unchanged in the hearts of HF-1b-conditional mutant mice (Fig. 4A). Interestingly, the expression of the NT-3 receptor, *trkC*, was dramatically decreased in NC-KO animals (Fig. 4A).

To determine if the decrease in *trkC* results in innervation deficiencies, we performed immunohistochemistry in the AVN using the neuronal marker Tuj-1 (β-III tubulin). In NC-KO animals, Tuj-1 immunostaining was substantially decreased

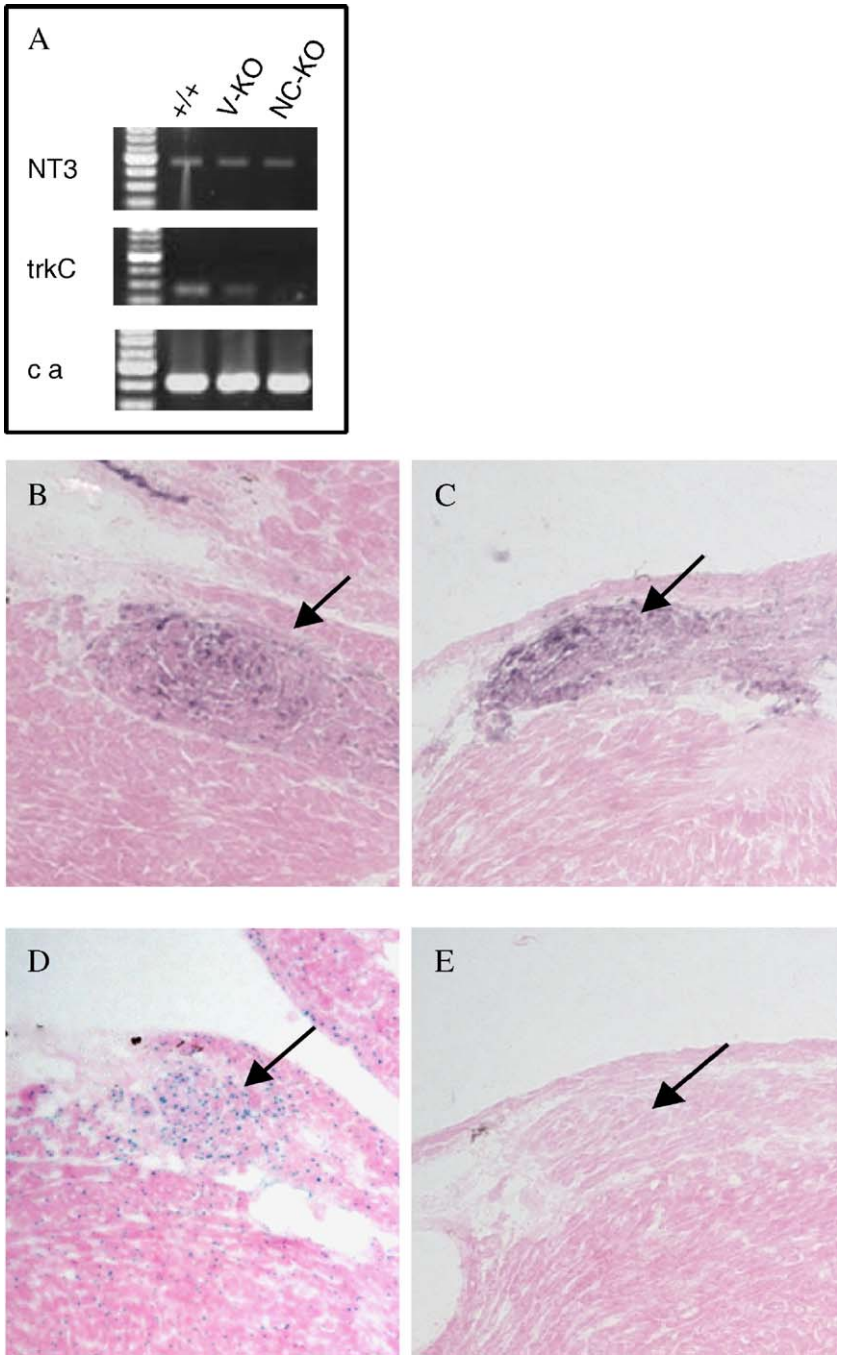

Fig. 4. HF-1b defect in the neural crest results in reduced TrkC expression. (A) RT-PCR for *NT-3* and *trkC* in the conditional knockout lines. Cardiac actin (ca) was used for the control. (B–E) Serial sections of wild type and NC-KO adult hearts. (B and C) Acetylcholinesterase (AChE) staining in wild type and NC-KO animals respectively to confirm the localization of the AV node. (D and E) Neuronal Class III β-tubulin immunohistochemistry staining in wild type and NC-KO hearts using β-galactosidase conjugated antibodies.

(Figs. 4D and E), suggesting abnormal innervations to the AVN region.

Discussion

The intrinsic requirement for HF-1b in the myocyte is important for the organization of the peripheral Purkinje network but is not necessary for function of the cardiac conduction system

While the HF-1b deficiency in the V-KO animals affects all components of the conduction system, the intrinsic requirement for HF-1b in the myocyte is restricted to the Purkinje network leading to a disorganization of Cx40 expression (Nguyen-Tran et al., 2000). Here, we have demonstrated that CX40 disorganization does not directly correlate with conduction system function as shown by the lack of ventricular electrophysiological phenotype and the absence of ventricular tachycardia or cardiac sudden death.

In other components of the conduction system, particularly the AVN and SAN, the intrinsic role for HF-1b is not crucial for propagation of the electrical impulse. It has been show recently that the transcription factor Nkx2.5 is crucial for AVN cell survival (Pashmforoush et al., 2001). Ventricular restricted knockout of Nkx2.5 leads to degeneration of the AV node and fatty infiltration of the central conduction system. However, we have not observed differences in NKX2.5 expression in the HF-1B mutant mice (not shown). Therefore, though all conduction system lineages are derived from the same cell type, each component has unique requirements necessary for its individual cell survival and provides further evidence for heterogeneity among all components of the conduction system.

MLC2v is never expressed in atrial myocytes, and yet, most of the phenotype observed requires abnormalities in the atrial segment. We attribute this finding to the expression of MLC2v in atrial portions of the conduction system, as reflected by in situ hybridization and LacZ positive signal in the SAN. The detection of MLC2v in the SAN was possible through the higher sensitivity and resolution of the nuclear LacZ staining, which was subsequently confirmed by in situ hybridization. Using only in situ hybridization would have probably escaped our detection of MLC2v in the SAN, due to the high possibility of artifact probe trapping in that area.

The extrinsic requirement for HF-1b in the neural crest is necessary for maturation and function of the central conduction system

While the neural crest cells do not incorporate into the conduction system lineages, previous data have suggested a

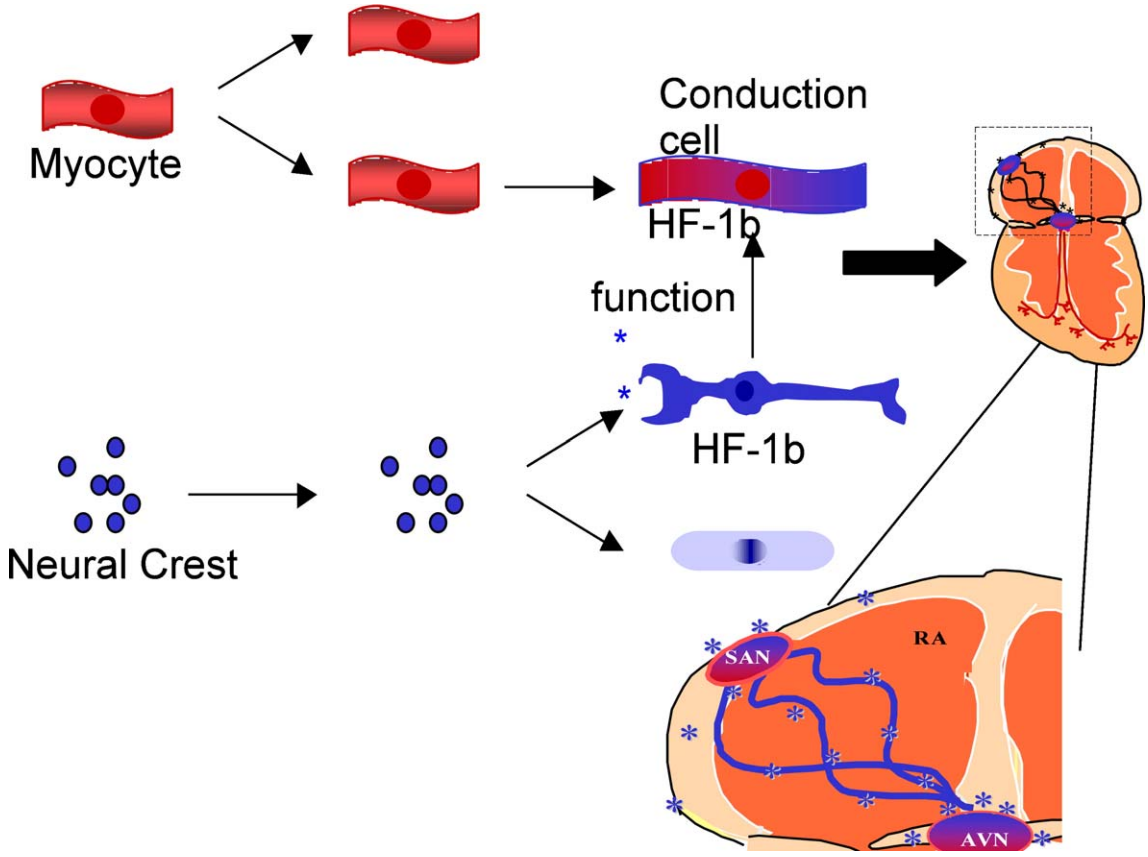

Fig. 5. Model for HF-1B action in the cardiac conduction system. Cardiac conduction system lineages are derived from the common myocyte. Neural crest cells, on the other hand, differentiate into neurons and smooth muscle cells of the aortic wall. HF-1b expression in the myocyte is necessary for the organization of peripheral conduction system cells. Signals from the nearby neural crest derived neurons, which also express HF-1b, are necessary for the final maturation and function of the atrial and atrioventricular components of the conduction system. SAN, sinoatrial node; AVN, atrioventricular node; RA, right atria.

role for neural crest in conduction system maturation and function (Poelmann and Gittenberger-de Groot, 1999). In the neural crest restricted HF-1b mutant mouse, electrophysiological analysis indicates that extrinsic cues from nearby neural crest or neural crest-derived tissues play a role for AV conduction system function. Our program stimulation data reveal significant AV node abnormalities in the NC-KO animals. AERP intervals were also elongated in the NC-KO animals suggesting additional atrial dysfunction that can be related either to a direct effect of neural crest cells or to defective postganglionic innervation, as suggested by the differential expression of TrkC in the HF-1B mutants.

During development, *trkC* is expressed in the migrating neural crest and later in the neural crest-derived neurons. Therefore, the downregulation of *trkC* preferentially in the NC-KO animals suggests that trkC is a downstream target of HF-1b in the neural crest. Neuronal β-tubulin immunohistochemistry confirms the reduced innervation in the AV node of NC-KO animals. However, since these innervation defects do not result in changes in heart rate variability the lack of innervation appears to be in the final step of neuronal patterning, we postulate that in the NC-KO model, the innervating neurons are not able to send their axons to target cells within the AV node and are thus unable to monitor conduction system function when the heart is subject to induced stimulation or stress.

Dual role for HF-1b in the heart

The intrinsic role for HF-1b in the myocyte is restricted to the peripheral conduction system, while the most prominent role for HF-1b in the neural crest appears in the central conduction system. This suggests a supplementary role for HF-1b in the two cell lineages, both of which are necessary for complete and uninterrupted cardiac function. The dual role for HF-1b in the myocyte and the neural crest provides a new model to describe the development and maturation of the cardiac conduction system (Fig. 5). Neither the V-KO nor the NC-KO recapitulated the complete phenotype seen in the global knockout. Conditional knockouts described here present varying degrees of disorganization of the gap junction Cx40 and conduction defects but, in contrast to the systemic mutants, both conditional knockouts display normal *MinK* expression in the epicardial myocardium (data not shown), suggesting that the failure of *MinK* downregulation in the global knockout may play a role in the development of sudden cardiac death. Further studies are aimed to address the effect of MinK overexpression on sudden death, although we cannot rule out the possibility of some unidentified factor to be responsible for the sudden death of the global KO mice. Therefore, the intrinsic role of HF-1b in the myocyte must be supplemented by an extrinsic requirement for HF-1b in the neural crest-derived cells, particularly the innervating neurons. The requirement for HF-1b in the neural crest is necessary at later stages for conduction system maturation and operation. In the absence of HF-1b, *trkC* levels are reduced, and the cardiac ganglia are not able to extend their axons to target cells of the AV node. As a result, these hearts are more susceptible to EP stimulation.

Conclusion

Our results suggest that the transcription factor, HF-1b, works through both the myocyte and neural crest lineages, providing intrinsic and extrinsic cues respectively, which are necessary for development and function of the conduction system. Neither the NC-KO nor the V-KO animals alone provide the complete phenotype seen in the HF-1b global knockout. Nevertheless, our data indicate that both neural crest and myocardial expression of HF-1B are required for proper cardiac conduction function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.10.018.

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